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(56) Documents Cited
Jap.J.Vet.Res. 1972,20(1-2),19-30 Infect.Immun. 1974, 10(4),750-756 Appl.Environ.Microbiol. 1977, 33(4), 963-966

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(54) Process for producing crystalline type A botulinum toxin

(57) A process for producing a crystalline type A botulinum toxin suitable as a therapeutic medicine such as for treating strabismus and blepharospasm comprises subjecting the botulinum type A toxin to removal of nucleic acid without denaturing the toxin by using a nucleic acid removing agent e.g. comprising a natural substance such as chitoxan. The toxin is obtained without substantial denaturation and/or deactivation.

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PROCESS FOR PRODUCING CRYSTALLINE TYPE A BOTULINUM TOXIN

This invention relates to a process for producing crystalline type A botulinum toxin which is suitable as a therapeutic medicine and, more specifically, it relates to a process for producing crystalline type A botulinum toxin utilising a nucleic acid removing agent, e.g. a natural substance such as, for example, chitoxan (trade name of products manufactured by Dainippon Seiyaku Co.).

Botulinum toxin, as a typical example of bacteria-derived neurotoxin is a proteotoxin produced from Clostridium botulinum. Botulinum toxin is classified into eight sero types of A, B, C1, C2, D, E, F, G depending on the antigenicity. Among them the type A toxin was the first one which was purified and crystallized (Lamnna, C.O.E.McElroy and H.W. Eklund: Science, 103: 613-614, 1946).

Heretofore, the production process for crystalline botulinum type A toxin has required complicated procedures conducted in the following order: acid precipitation (1), water washing, extraction with calcium chloride and acid precipitation (2), extraction with phosphate buffer, precipitation with ethanol and extraction and deposition in ammonium sulfate solution. In addition, since the production operation is complicated, it requires the skill and perception of experienced engineers. Further, the process gives only a low toxin recovery rate and one cannot expect to obtain a required and effective amount thereof. Moreover, although a method comprising combination of, for example, a protamine treatment, ion exchange

chromatography and gel filtration has also been adopted, nevertheless crystallization efficiency of such methods is not optimal.

One of the problems involved in the crystallization of botulinum type A toxin is how to obtain a toxin of an easily crystallizable macro molecule in good yield. Another of the problems is how to enable elimination of crystallization inhibiting substances mainly comprising nucleic acid contained in the culture solution without denaturing the toxin. The first problem can be solved by selecting a strain yielding botulinum type A toxin having a molecular weight of 500,000 to 900,000 and the solution to the second problem depends on how the nucleic acid can be removed under mild conditions.

The present invention is directed to the foregoing disadvantages in the prior art and an object of the invention is to provide a process for producing crystalline type A botulinum toxin having improved stability and effectiveness as a therapeutic medicine such as a medicine for the treatment of strabismus and blepharospasm, by obtaining a pure toxin.

Thus, in one aspect the present invention provides a process for producing a crystalline Type A botulinum toxin, which process comprises the step of removing nucleic acid using a nucleic acid removing agent, whereby to produce crystalline type A botulinum toxin substantially without loss of the toxicity thereof. With such a process, the botulinum type A toxin is crystallized substantially without loss of its toxicity. The toxin is obtained substantially without denaturation and deactivation, in a substantially pure form, in an improved yield for toxic activity. The product is suitable as a therapeutic medicine, e.g. to treat strabismus or blepharospasm.

Botulinum type A toxin is crystallized without loss of toxicity by removing, during the process, nucleic acid with a nucleic acid removing agent. The nucleic acid removing agent preferably comprises a natural substance such as chitoxan.

First, among strains yielding the botulinum type A toxin, strains producing L toxin of molecular weight 500,000 and LL toxin of molecular weight 900,000 with high hemoagglutination titer and showing high toxicity are conveniently selected. The strains are conveniently cultured in an appropriate culture medium, for example, NZ-amine culture medium, an acid is added to a culture-filtrate to cause precipitation, and a phosphate buffer solution with the molar concentration being changed is used instead of calcium chloride to extract the toxic ingredient effectively from the precipitate. Further, the liquid extract is repeatedly subjected to pH control, overnight standing, centrifugation, dissolution in an acetate buffer solution, pH modification and centrifugation to obtain a clear supernate. A nucleic acid removing agent is added to the supernate under most effective conditions.

Heretofore, calcium and alcohol have been used customarily for removing nucleic acid. However, no remarkable result can be obtained even when conditions such as concentration and pH of the nucleic acid removing agent are changed. Further, protamine has been used as a nucleic acid removing agent but it causes precipitation of not only nucleic acid but also necessary proteins though it has an excellent effect as a removing agent. In addition, it brings about the problem of differences in the homogeneity between batches, which is a fatal defect of natural products. Further, it is necessary to use an additional procedure for eliminating excess protamine from the resultant

precipitate. On the other hand, chitoxan (trade name products manufactured by Dainippon Seiyaku Co.) is a natural high molecular weight substance, which is a natural substance obtained by deacetylating chitin (poly-N-acyl-β-D-glucosamine) contained in outer shells, for example, of crabs and shrimps and C-7, C-9 obtained by reducing the molecular weight thereof can effectively remove nucleic acid without loss of the toxin. When ammonium sulfate salting out is conducted after removal of nucleic acid with chitoxan, excessive chitoxan does not precipitate and, after dissolving the salted out toxin into a phosphate buffer solution, ammonium sulfate is conveniently gradually added and stood still to easily crystallize the toxin.

In the production process using Duff's method, the yield of the crystalline type A botulinum toxin was as low as 1.4 - 3.1 assuming the culture solution as 100. Although it could be increased up to about 5% by improving the extraction method, the yield of the toxic activity can be increased to 10% by the method according to the present invention, particularly using chitoxan.

According to the present invention, nucleic acid can be removed efficiently when producing the crystalline type A botulinum toxin. The yield of the toxic activity can be improved and, as a result, a remarkable effect can be attained as a therapeutic medicine such as for treating strabismus and blepharospasm.

Other features and advantages of the present invention will become apparent from the following Example:

Example

(Culturing)

Clostridium botulinum, strain Hall, NZ-amine culture medium, standing culture at 35°C for 4 days.

(Acid precipitation 1)

Sulfuric acid added: pH adjusted to 3.5 with 3N H_2SO_4 stood still overnight (4°C)
Supernate removed (siphon)
Centrifugation
Precipitate: Dissolved in distilled water, pH adjusted to 5.0 with 0.1N NaCl
Centrifugation

(Extraction)

Precipitate: 0.2M phosphate buffer solution (pH 6.0) added
Centrifugation
Supernate
Precipitate: 0.5M NaCl incorporated 0.1M tris-HCl buffer solution (pH 7.5) added
Centrifugation

(Acid precipitation 2)

HCl added: pH adjusted to 3.7 with 2N HCl, stood still overnight (4°C)
Supernate removed (siphon)
Centrifugation
Precipitate: Dissolved in 0.05M acetate buffer solution (pH 5.0)
Centrifugation

(C-9 treatment)

Supernate: 5% C-9 solution added by 0.1% for one min, stirred for 10 min in an ice box
Centrifugation
Supernate

Ammonium sulfate added: Solid ammonium sulfate added to make 50% saturation solution. Stood still overnight at 4°C.
Centrifugation
Precipitate: Precipitate dissolved in 0.03M phosphate buffer solution at pH 6.8
Dialysis: Dialyzed with 0.03M phosphate buffer solution with pH 6.8

(Crystallization)

Ammonium sulfate added : Ammonium sulfate added until the solution exhibits pearlescent color, providing that the final concentration does not exceed 0.9M concentration
Crystal deposition: Left at 4°C

[(Preparation of 5% C-9 solution)

Distilled water was added to C-9 (Kurimover, manufactured by Dainippon Seiyaku, Lot No. K143) at 10 to 1 ratio. Further, 1N HCl was added at a ratio of 2 and stirred by a stirrer for 2 hours. pH was adjusted with 1N sodium hydroxide to 4-6. The solution was diluted with distilled water to a 5% solution.]

CLAIMS

1. A process for producing a crystalline type A botulinum toxin, which process comprises the step of removing nucleic acid using a nucleic acid removing agent, whereby to produce crystalline type A botulinum toxin substantially without loss of the toxicity thereof.

2. A process as claimed in claim 1 wherein said nucleic acid removing agent is a natural substance.

3. A process as claimed in claim 1 or claim 2 wherein said nucleic acid removing agent is chitoxan.

4. A process as claimed in any one of the preceding claims wherein said type A botulinum toxin is produced from a strain of bacteria yielding botulinum type A toxin having a molecular weight of 500,000 to 900,000.

5. A process as claimed in any one of the preceding claims, which process comprises the steps of culturing a strain of bacteria yielding botulinum type A toxin, precipitating a culture filtrate with an acid, extracting the precipitate with a phosphate buffer solution, adjusting the pH of the liquid extract, standing the adjusted solution overnight, centrifugally separating the adjusted solution, dissolving the separated precipitate with an acetate buffer solution, modifying the pH value of the dissolved solution, centrifugally separating the modified solution and adding said nucleic acid removing agent to the supernate.

6. A process for producing a crystalline type A botulinum toxin substantially as herein described with reference to the Example.

7. Crystalline type A botulinum toxin when made by a process as claimed in any one of claims 1 to 6.

8. A pharmaceutical composition comprising a crystalline type A botulinum toxin as claimed in claim 7 together with at least one pharmaceutical excipient.

Patents Act 1977 Examiner's report to the Comptroller under Section 17 (The Search report)	Application number GB 9321794.1
Relevant Technical Fields (i) UK Cl (Ed.L) C3H (HC2) (ii) Int Cl (Ed.5) C07K 15/04; C12P 21/00	Search Examiner C SHERRINGTON Date of completion of Search 17 JANUARY 1994
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASES : WPI, CLAIMS, DIALOG/BIOTECH	Documents considered relevant following a search in respect of Claims :- 1 TO 8

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
A	Jap.J.Vet.Res. 1972, 20(1-2), 19-30 Purification and crystallisation of Clostridium botulinum Type C toxin	1
A	Infect.Immun.1974, 10(4), 750-756 Purification and Some Properties of Progenitor Toxins of Clostridium botulinum Type B	1,2
X	Appl.Environ. Microbiol 1977, 33(4), 963-966 Improved Procedure for Crysallization of Clostridium botulinum Type A Toxic complexes	1

Databases:The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).

Departments, Programs, and Services

Admitting
Anesthesia
Arthritis Program
Assistive Technology Department
Brain and Spinal-Cord Injury Program
Cast Room
Cerebral Palsy Program
Child and Family Services
Chronic Pain Program
Craniofacial Program
Dentistry
Epilepsy Unit (4 North)
Facsimile Service
Family Resource Center
Foundation
Gillette Technology Center
Human Resources
Information Desk
Information Systems
Marketing and Marketing Communications
Medical Records
Medical Staff Office
Medical/Surgical Unit (4 South and 4 West)
Motion Analysis Laboratory
Neurodevelopmental Pediatrics
Neurology Program
Neuromuscular Clinic
Neurosurgery Services
North Clinics
Occupational Therapy
Operating Room
Orthopaedics Program

- Limb Length Service
- Spine Service
- Arm and Hand (Upper Extremities)
- Brachial Plexus
- Prosthetics
- Hip Service
- Leg and Foot (Lower Extremities)

Outpatient Clinic
Outreach Program
Pediatric Intensive Care Unit
Peri-Anesthesia Services
Photography
Physical Therapy
Quality Improvement Resources
Radiology
Rehabilitation Therapies
Rehabilitation Unit (4 North)
Research
Residency
Respiratory Care

Rhizotomy Service
Speech/Language Pathology Services
Spasticity Evaluation Service
Spina Bifida Program
Spinal-Cord Injuries
Sports Medicine Program
Urology
West Clinic

Admitting

Outpatient Services

Appointments for new patients can be scheduled for our Outpatient Clinic, Gillette West Clinic, Assistive Technology Department, Child and Family Services and Rehabilitation Therapies by calling Admitting at: **(651) 229-3944** or **(651) 229-3851.**

Appointments for the Gillette West Clinic, the Gillette Technology Center (including outreach visits), the Assistive Technology Department and the Gillette North Clinics can also be made by calling these departments or locations directly.

Gillette West Clinic	(952) 936-0977
Gillette Technology Center	(651) 636-9443
Assistive Technology Department	(651) 229-3800
Gillette North Clinics	(218) 728-6160

Appointments for existing patients can be made by calling:	(651) 290-8707

Inpatient Admissions

Physicians may admit patients to Gillette by calling Admitting. Any questions regarding admitting policies should be directed to the admitting manager.

Lynn Carpentier, admitting manager	(651) 229-3845
Admitting	(651) 229-3848

Anesthesia

Our Anesthesia staff is specially trained to meet the surgical and anesthesia needs of children who have disabilities.

Gwen Shuller-Bebus, R.N., nurse manager	(651) 602-3268

Lynn Christianson, M.D., medical director	(651) 229-3994
Quality Nurse Anesthesia Professionals Corp.	(651) 229-3864

Arthritis Program

The Arthritis Program treats children and adolescents who have a wide range of rheumatologic conditions. Using a multidisciplinary team approach, the program offers comprehensive diagnostic evaluations and the newest available treatment options.

Richard K. Vehe, M.D., medical director	(651) 229-3870

Assistive Technology Department

The Assistive Technology Department (ATD) designs and customizes orthoses, powered wheelchair controls, specialized seating, communication devices, prostheses, protective headgear and wheelchairs. The staff works with physicians and therapists to evaluate the individual needs of each client. The department accepts direct referrals from physicians, schools and community agencies. For more information about a specific service, call the appropriate manager or supervisor.

Linda Valeri, ATD manager	(651) 229-3808
Kathy Molina, extremity orthotics supervisor	(651) 229-3807
Jim Cepress, seating and adaptive equipment supervisor	(651) 229-3804
Richard Stricker, prosthetics supervisor	(651) 229-3809
Paul Swanlund, spinal orthotics supervisor	(651) 229-3811
Gary Kroll, technician supervisor	(651) 312-3117

Brain and Spinal-Cord Injury Program

The Pediatric Brain and Spinal-Cord Injury Program offers comprehensive inpatient and outpatient pediatric rehabilitation for children and teenagers with brain or spinal-cord injuries. The program includes evaluation, treatment, discharge planning and follow-up. Services include: pediatric physical medicine and rehabilitation; pediatric neurology; speech/language pathology; physical and occupational therapy; psychological assessment and counseling; educational programming; family support and education; and equipment and assistive technology consultation.

Linda E. Krach, M.D., co-medical director	(651) 229-3819
Robert Kriel, M.D., co-medical director	(612) 347-2680
Diane Nelson, program manager	(651) 229-3877

Cast Room

The Cast Room provides casting services to children and young adults who have disabilities.

Thomas Leppla, orthopaedic physician's assistant	(651) 229-3882

Cerebral Palsy Program

Using the expertise of a multidisciplinary medical team, our Cerebral Palsy Program evaluates and treats children, adolescents and young adults who have cerebral palsy. Computerized gait analysis is often used to help pinpoint areas of concern and plan appropriate treatments. Contemporary treatment options include botulinim toxin (BOTOX®, MYOBLOC™), intrathecal baclofen, selective dorsal rhizotomy, orthopaedic surgery, bracing and therapy.

For information on related services, see the Rhizotomy and Spasticity Management sections.

James Gage, M.D., medical director	(651) 229-3840
Candace Vegter, M.A., CCC, program manager	(651) 290-8712